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*Ray S. Kestis* 7/27/99  
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## INTRODUCTION

Ataxia telangiectasia (AT), which results from mutations in both copies of the *ATM* gene, is relatively rare with an incidence of approximately only 1 in 100,000. However, the heterozygous condition which is characterized by a mutation in one copy of the gene, is thought to affect roughly 0.5-1% of the population. These individuals do not suffer from AT, however, they appear to be particularly susceptible to the induction of breast cancer by radiation. The hypothesis being examined in this project is that women who are *ATM* gene carriers and receive radiation therapy, generally as part of a breast conservation treatment, are at increased risk of developing a second primary breast cancer. Hence, the goal of this study is to determine whether radiation exposure resulting from the scattered dose to the contralateral breast is associated with an increased occurrence of bilateral breast cancer among women who are heterozygous for *ATM*. This will be accomplished through screening bilateral breast cancer patients for *ATM* mutations. Confirmation of this hypothesis would have important and direct implications upon patient care in that it would suggest that breast cancer patients should be tested for *ATM* heterozygosity. If found positive, a possible recommendation may be that these women should not be candidates for radiotherapy and other treatment modalities utilized. Alternatively, a more effective block technique with dose reduction to the contralateral breast may be implemented for these patients. This would have the positive impact upon the vast majority of patients that are not *ATM* carriers as they could be assured that their risk for radiation induced breast cancer is extremely low as *ATM* carriers may account for most breast cancers resulting exposure of the contralateral breast to radiation associated with radiotherapy.

## BODY

**Task 1: Month 1: Identify with Dr. Paul Tartter the bilateral breast cancer patients who fall into the two categories to be used in this study.**

This was accomplished using records Dr. Tartter has assembled for bilateral breast cancer patients treated at Mount Sinai over the past 18 years.

**Task 2: Month 1: Establish a procedure by which the breast cancer patients will be recruited into the study.**

There was relatively easy accessibility to unilateral breast cancer patients as many of these patients were treated in the Radiation Oncology Department and continue to come to Mount Sinai periodically for follow-up visits. Arrangements were made so that when patients selected for this study arrived for their exam, an investigator was available to explain the project to the patients and obtain informed consent for participation in the study following which a nurse would draw a blood sample. A difficulty arose, however, recruiting bilateral breast cancer patients into the study as many of these patients, who were treated as long as 18 years ago, had either died, been lost to follow up or rarely visited Mount Sinai. Therefore, an alternate strategy was developed to access the bilateral patients in which paraffin embedded tissue, preferably lymph node biopsy samples, were obtained for these individuals. A collaboration was established with Dr. Ira Bleiweiss, the pathologist at Mount Sinai responsible for review of all breast cancers. Using this approach, we successfully retrieved paraffin-embedded tissue blocks from 41 bilateral patients identified by Dr. Tartter.

**Task 3: Month 1: Decide which regions of ATM will be subjected to analysis based upon results available in the literature at the time the project begins**

As of this point, there are no specific allelic hotspots or regions of *ATM* which have been identified for mutations. Therefore, the entire coding region of the gene is being examined.

**Task 4: Months 2-32: Obtain blood samples from breast cancer patients**

Blood samples were obtained from a total of 37 unilateral breast cancer patients. In addition, tissue blocks were retrieved for 41 bilateral breast cancer patients.

### **Task 5: Month 2: Create appropriate primers for RT-PCR and sequencing**

During the period when a procedure to recruit bilateral breast cancer patients into the study was being established, efforts were also initiated to create primers for RT-PCR. As this effort was well underway by the time a decision was made to use genomic DNA obtained from paraffin embedded tissue, it was decided to complete construction of the cDNA primers and use them to screen for *ATM* mutations in unilateral breast cancer patients. The results of this portion of the project have been submitted for publication and a copy of this manuscript entitled "*ATM* heterozygosity and breast cancer: screening of 37 breast cancer patients for *ATM* mutations using a non-isotopic RNase cleavage-based assay" has been included in the appendix to this report.

Work was accomplished during the past year in which primers were designed and validated to amplify the exons which comprise the *ATM* gene. The sequences of these primers are listed in Tables 1 and 2.

### **Task 6: Months 3-36: Isolate mRNA from blood lymphocytes of patients**

As described, rather than isolating mRNA from blood samples, genomic DNA was isolated from paraffin-embedded biopsy tissues. To accomplish this, the ONCOR EX-WAX™ DNA Extraction Kit was used. The procedure employed was to first cut 10 sections of 5  $\mu$  thickness from a paraffin-embedded lymph node biopsy sample and place them in a 1.5 ml tube. 1 ml of 100% ethanol was added, vortexed 15 sec, centrifuged at 12,000 rpm 3 min, the ethanol removed and the pellet air dried. 150  $\mu$ l of digestion solution and 50  $\mu$ l of protein digesting enzyme solution were added and the tube incubated 4-18 hr at 50°. 100  $\mu$ l of extraction solution was mixed and after 15 sec the tube was centrifuged 10 min at 12,000 rpm. The supernatant was removed by poking the pipette tip through the paraffin layer on top and withdrawing the supernatant, leaving the paraffin and pellet behind. 150  $\mu$ l of precipitation solution was added to the supernatant, the tube inverted 3 times, 900  $\mu$ l of 100% ethanol (-20°) added and mixed. After 1 hr the tube was centrifuged for 10 min at 12,000 and the supernatant discarded. The pellet was air dried and 50  $\mu$ l of resuspension solution added for 1 hr at 50°.

**Task 7: Months 3-36: Perform RT-PCR with these samples to amplify in sections the coding region of the *ATM* gene.**

RT-PCR was not necessary as genomic DNA samples were used. The first stage PCR reactions were performed using approximately 100-500 ng genomic DNA, 125  $\mu$ M dNTPs, 250 nM primers, 1 U Taq polymerase in a buffer containing 10 mM Tris.Cl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 0.01% gelatin and RNase free water to a total volume of 50  $\mu$ l. The PCR reactions were performed in a Perkin Elmer 9700 Thermal Cycle at 94° for 4 min, followed by 30 cycles of 1 min at 94°, 2 min at 54° and 1.5 min at 72° and one cycle for 8 min at 72°. 10  $\mu$ l of the first stage PCR product was used in a second series of PCR reactions using the same conditions to achieve further amplification of the region of interest and to add the T7 and SP6 promoters to the PCR products.

**Task 8: Months 3-36: Use NIRCA to identify PCR products containing mutations**

The 41 patients for whom exons 3, 4, 15 and 24 of *ATM* were amplified using PCR have been examined for mutations using a Non-radioisotopic RNase Cleavage-based Assay (NIRCA). Each second stage PCR product was transcribed in two reaction mixes using 2  $\mu$ l of the PCR product, 250 nM rNTPs, 2 U of either T7 or SP6 RNA polymerase in a buffer containing 40 mM Tris.Cl, pH 7.5, 7 mM MgCl<sub>2</sub>, 2 mM spermidine, 25 mM NaCl and 10 mM dithiothreitol and brought to a final volume of 10  $\mu$ l with RNase free H<sub>2</sub>O and incubated for 1 hr at 37°. 10  $\mu$ l of buffer containing 80% (v/v) formamide, 25 mM NaCl, 2 mM EDTA, pH 8.0 was added to each transcription reaction. The T7 and SP6 reactions were mixed, incubated at 95° for 3 min and allowed to cool at room temperature for 1 min. 4  $\mu$ l of the duplex reactions were mixed with 16  $\mu$ l of RNase digestion buffer (10 mM NaCl, 10 mM Tris.Cl, pH 7.5, 1 mM disodium EDTA, pH 8.0) with 0.5  $\mu$ g/ml RNase A, RNase I and RNase T1 and brought to a final volume of 20  $\mu$ l. The reaction mixtures were incubated at 37° for 20 min and resolved on a 3% agarose gel run at 100 V for approximately 45 min.

**Task 9: Months 3-36: Sequence all PCR products which appear to exhibit mutations**

There was no evidence of any mutations in the samples that have been analyzed up to this point and therefore it has not been necessary to perform any DNA sequencing.



TABLE 1. SEQUENCES OF FIRST STAGE *ATM* GENOMIC PRIMERS

Fragment Number	Primer Sequence
2-Sense	ATTACTGTGTTTTTGTTCCT
2-Antisense	TGCTCATTCACTGATAGATG
3-Sense	TTTTTCACACCTCTTTCTCTC
3-Antisense	ATAATAATGGGTTACTAATCAC
4-Sense	TGAAATGTGTGATTAGTAAC
4-Antisense	GAAAAATAAAGACAGTAAAAT
5-Sense	CATTTTGTTTTCTTGAA
5-Antisense	GCCATTTATTTATAATTTAT
6-Sense	GTTGCCATTCCAAGTGTCTTA
6-Antisense	CAAACAACAACCTTCAAACA
7-Sense	CTGTATGGGATTATGGAATA
7-Antisense	CAAAGAAAAAGAGATTAGATT
8-Sense	TTGAGCTTGTTTGTTTCTTC
8-Antisense	AGACTTCTATGTTTGAATGA
9-Sense	CTAGCAGTGTAACAGAGTA
9-Antisense	ATAGGCTTTTTGTGAGAACA
10-Sense	GCAACAACAGCGAACTCTG
10-Antisense	GAATGAGAAAATGGTAACACTT
11-Sense	ATGTGATGGAATAGTTTTCAA
11-Antisense	AGTAACAAACTATGAAAATGA
12-Sense	TTTCACAATTGTCCTTTGTT
12-Antisense	GCAGCATGCTAATGAACTTA
13-Sense	CTAAGTGAAGCTTTTTGTTT
13-Antisense	GATAACATACCATTCTGG
14-Sense	CATATAAGGCAAAGCATTAG
14-Antisense	AGTTTACCAAAGTTGAATCATA

15-Sense	ATATTTTTATTTGTGGTTT
15-Antisense	TTAGTATAAATTCTCCTACG
16-Sense	TTGCATTTTTCCTTCTATTCA
16-Antisense	CTCCAGCCTGGGTGACAGAGA
17-Sense	ACACTGTCTGCCGAGAATAAT
17-Antisense	AGCAAAACAGGAAGCATACTT
18-Sense	CTCCTGCAAGAAGCCATCT
18-Antisense	AAGAAATCCCAAGTAGTAAAT
19-Sense	GTTGTGCCCTTCTCTTAGTGTT
19-Antisense	ACTCATTACATTTAGTCAGCAA
20-Sense	CCCTCCTACCATCTTAG
20-Antisense	TAAGGTTTTAATTTCTTACG
21-Sense	TAAAATAACTGATGTGTTCTGTT
21-Antisense	CAAACTTGCATTTATC
22-Sense	TTTAACTTTGGAAAACCTTAC
22-Antisense	ATGCAAAAACCTCACCT
23-Sense	TCTTTGTTTGTTAATGAGTA
23-Antisense	CAGCATTCCAAATACTTCAT
24-Sense	GTTTGTTTGCTTGCTTGTTT
24-Antisense	ATTTATGGGATATTCATAGC
25-Sense	TGGAGTTCAGTTGGGATTTTA
25-Antisense	TTCACAGTGACCTAAGGAAGC
26-Sense	CTTAACACATTGACTTTTTGG
26-Antisense	GTATGTGTGTTGCTGGTGAG
27-Sense	CCTGTATATTTTAAATTTT
27-Antisense	GAAGCCATACCTGTT
28-Sense	AGATTGTGGTGGAGTTATTGA
28-Antisense	TTAAAAAGAGTGATGTCTATA

29-Sense	TTAAAACGATGACTGTATT
29-Antisense	GAAGGAATGTTCTATTATTA
30-Sense	CCGAGTATCTAATTAAACAAG
30-Antisense	CAGGATAGAAAGACTGCTTAT
31-Sense	TTTTTCTCCCTATATTAGGC
31-Antisense	TACCATTTTGAAGATGAGTC
32-Sense	AGTTTTGTTGGCTTACTTTA
32-Antisense	GAGCATTACAGATTTTTTGAA
33-Sense	GTGTTAAAAGCAAGTTACATT
33-Antisense	AAGAAACAGGTAGAAATAGC
34-Sense	GCAATTATAAACAAAAAGTGT
34-Antisense	TATATGTGATCCGCAGTTGAC
35-Sense	CAGCATTATAGTTTTGAAAT
35-Antisense	GTGTGAAGTATCATTCTCCA
36-Sense	TGGTGTACTTGATAGGCATTT
36-Antisense	GACCCACAGCAAACAGAACTG
37-Sense	TTTACATTTTCTAATCCCT
37-Antisense	AATCTTCTTACTTCACACAT
38-Sense	CATTTTTACTCAAACATTG
38-Antisense	TCTTAAATCCATCTTTCTCTA
39-Sense	GGA CTGAGGGGAGATATTTT
39-Antisense	CGTAAGAAGCAACACTCATT
40-Sense	CAACATGCTTTTATTTTGATA
40-Antisense	TATATACCCTTATTGAGACAA
41-Sense	TCTCATTTAAAAGAGGTGTTCT
41-Antisense	TGAGATAAATACTGTCATAAA
42-Sense	TTCAATTTTTGTTGTTTCCAT
42-Antisense	TTACTATACCGTTGTTTAGAA

43-Sense	CCCAAAGCTATTTTCACAAT
43-Antisense	GCTTTACCAAATCTGGGAA
44-Sense	AAAAACAAAATAACTCCTGT
44-Antisense	ATCAAGTCAAATTTCTTACC
45-Sense	TTTTTCTTTGACTTATCTCA
45-Antisense	ACAGTAACTTTGTCTTTTCA
46-Sense	TTCAGAGTGTCTTTTCTTTT
46-Antisense	AGGATAACAAAGTCATACGA
47-Sense	ACATGAACTCTATGTCGT
47-Antisense	GATGACATAGTTTTAAATTG
48-Sense	TATTCCCATATGTCATTTTC
48-Antisense	CACTAATCCAGCCAATAAA
49-Sense	CCGTACATGAAGGGCAGTTGG
49-Antisense	TTGATGAAAAGATGAAGCATA
50-Sense	TAATTTAAATTGGTTGTGTT
50-Antisense	TTGTCACTTGTAGAAACCTA
51-Sense	GTTTCATGGCTTTTGTGTTTT
51-Antisense	TAGAATATTGGGCTGAGTAAC
52-Sense	ACTTGCTTAGATGTGAGAATA
52-Antisense	GTTTGATTTTCAGGTTTACTT
53-Sense	AAATCTAATAGTTCTTTTCTT
53-Antisense	CTGAATATCACACTTCTAAA
54-Sense	GGGTAGTTCCTTATGTAATGT
54-Antisense	TAACACAGCAAGAAAGTAAC
55-Sense	AGTTTGTCACTAAAATCTCTTC
55-Antisense	AATGGTTCAATCTAATAAAG
56-Sense	GCAAATAGTGTATCTGACCTA
56-Antisense	TAAAACTCTAAGGCTAACCAG

57-Sense	TTTGCTATTCTCAGATGACTCT
57-Antisense	ATATGTTTTTGGTGAACAACTAAC
58-Sense	AGGTCAACGGATCATCAAAT
58-Antisense	TTAATTTTGGGTGTCACTC
59-Sense	TGCCCCTATATCTGTCATA
59-Antisense	CAAAGTGCTCAATCTACTAT
60-Sense	TAGAAAGAGATGGAATCAGTG
60-Antisense	TCTTGGTAGGCAAACAACATT
61-Sense	CCTCCTAACTTCACTGTATT
61-Antisense	TTCCCTCCTTTACTTCAT
62-Sense	CAGGCTCAGCATACTACACAT
62-Antisense	GACGAGATACACAGTCTACCT
63-Sense	ATGAAACTGGTTCTACTGTT
63-Antisense	AAATCTGAAAAACTGACAAC

TABLE 2. SEQUENCES OF SECOND STAGE *ATM* PRIMERS

Fragment Number	Primer Sequence
2-Sense	<u>TAATCGACTCACTATAGGGCAGAGGCATACATCACAAT</u>
2-Antisense	<u>ATTTAGGTGACACTATAGAAACTAAACAGATACAAATCC</u>
3-Sense	<u>TAATCGACTCACTATAGGGTTTTACAGACAGTGATGTG</u>
3-Antisense	<u>ATTTAGGTGACACTATAGAAAAGTAATTTACTACCTTTTCG</u>
4-Sense	<u>TAATCGACTCACTATAGGGAACCCATTATTATTTCTTT</u>
4-Antisense	<u>ATTTAGGTGACACTATAGAAGAATACCTAAAAACAGCATC</u>
5-Sense	<u>TAATCGACTCACTATAGGGCAGAAATATATTCAGAAAGA</u>
5-Antisense	<u>ATTTAGGTGACACTATAGAAACATCACTTACTTCTGTTT</u>
6-Sense	<u>TAATCGACTCACTATAGGGGTTTTTCTTTATTTGTTTAT</u>
6-Antisense	<u>ATTTAGGTGACACTATAGAAAAAATTCACAAACAACAACC</u>
7-Sense	<u>TAATCGACTCACTATAGGGTTTTTTAAGAATTGTTCTCTG</u>
7-Antisense	<u>ATTTAGGTGACACTATAGAACAAAAGAAAAAGAGATTAGA</u>
8-Sense	<u>TAATCGACTCACTATAGGGCAGTTGAGCTTGTTTGTT</u>
8-Antisense	<u>ATTTAGGTGACACTATAGAATTCAAAACAGTAAACATTTTC</u>
9-Sense	<u>TAATCGACTCACTATAGGGTGGATTACAGGTGCTTATG</u>
9-Antisense	<u>ATTTAGGTGACACTATAGAATTACTGTACCTGGTGACAGA</u>
10-Sense	<u>TAATCGACTCACTATAGGGTTTACAGGTTTTTAAATGAAG</u>
10-Antisense	<u>ATTTAGGTGACACTATAGAAACACTTTACCAAGGCAC</u>
11-Sense	<u>TAATCGACTCACTATAGGGTTTAGGCTACAGATTGCAAC</u>
11-Antisense	<u>ATTTAGGTGACACTATAGAAAGTCAGACATAATGCATGC</u>
12-Sense	<u>TAATCGACTCACTATAGGGTTATAGTCCTGCAGTATGCT</u>
12-Antisense	<u>ATTTAGGTGACACTATAGAAACCTGTGAAGAATTGGAG</u>
13-Sense	<u>TAATCGACTCACTATAGGGTTCTTTGTAGTAATTTTCCT</u>
13-Antisense	<u>ATTTAGGTGACACTATAGAAAATTCATTGCAGCTTTA</u>
14-Sense	<u>TAATCGACTCACTATAGGGTTAAAGATCTTACTTTCTTG</u>
14-Antisense	<u>ATTTAGGTGACACTATAGAAAATCTCACCTCAGATGA</u>

15-Sense	<u>TAATCGACTCACTATAGGGACTTTAAGATTACAAATTCA</u>
15-Antisense	<u>ATTTAGGTGACACTATAGAAGCTTTCTGGAATAATTCT</u>
16-Sense	<u>TAATCGACTCACTATAGGGTTTTCTTCTATTCCACAAT</u>
16-Antisense	<u>ATTTAGGTGACACTATAGAAAAGAAGAAGAAAATCTTACC</u>
17-Sense	<u>TAATCGACTCACTATAGGGGTTTGTCTTAATTGCAGAAG</u>
17-Antisense	<u>ATTTAGGTGACACTATAGAAAATATCTGCAATGTCATTCT</u>
18-Sense	<u>TAATCGACTCACTATAGGGGCATCCTTCATCAAAAA</u>
18-Antisense	<u>ATTTAGGTGACACTATAGAAATTTACCTATGGTACTTTGG</u>
19-Sense	<u>TAATCGACTCACTATAGGGTTAATGAGTGCTTTTTATTT</u>
19-Antisense	<u>ATTTAGGTGACACTATAGAAAGCTTCTTCATTTAACGTA</u>
20-Sense	<u>TAATCGACTCACTATAGGGTAATGCTTTTAAAGGAGCTT</u>
20-Antisense	<u>ATTTAGGTGACACTATAGAAAGTGGTTTCAGAAGTTCAAG</u>
21-Sense	<u>TAATCGACTCACTATAGGGTTTACCACAGCAATGTGT</u>
21-Antisense	<u>ATTTAGGTGACACTATAGAATAGGACCACAAAATAGACTG</u>
22-Sense	<u>TAATCGACTCACTATAGGGTTGATTTGCAGGCATCTAACA</u>
22-Antisense	<u>ATTTAGGTGACACTATAGAACAAGCAAAGTTTTAAGGCA</u>
23-Sense	<u>TAATCGACTCACTATAGGGCACAGTTCTTTTCCCGTAG</u>
23-Antisense	<u>ATTTAGGTGACACTATAGAATGAATATTTGACCCATTACC</u>
24-Sense	<u>TAATCGACTCACTATAGGGCTTGTTTTAAGATTGTTCCA</u>
24-Antisense	<u>ATTTAGGTGACACTATAGAAATTACCATTCTCTCATTCC</u>
25-Sense	<u>TAATCGACTCACTATAGGGTAAATTTCTTTTTAAGTCCC</u>
25-Antisense	<u>ATTTAGGTGACACTATAGAAATTTCAACAAGGTGAGGTT</u>
26-Sense	<u>TAATCGACTCACTATAGGGGTGCAGGTTTTAGAGA</u>
26-Antisense	<u>ATTTAGGTGACACTATAGAAAACTTACCTATAGAAATCC</u>
27-Sense	<u>TAATCGACTCACTATAGGGAGATCTTGTTATAAGGTTTT</u>
27-Antisense	<u>ATTTAGGTGACACTATAGAACCAATAAGTTTTCACTTT</u>
28-Sense	<u>TAATCGACTCACTATAGGGAATATATTTTAATTTTGTGC</u>
28-Antisense	<u>ATTTAGGTGACACTATAGAAAAGTTTAAAATGTACATACC</u>

29-Sense	<u>TAATCGACTCACTATAGGGTTTTCCCTTAACTCTGTTAG</u>
29-Antisense	<u>ATTTAGGTGACACTATAGAAAATGTATACTTACAGGGCTTT</u>
30-Sense	<u>TAATCGACTCACTATAGGGAAATCTGTTTATTTTCTAGG</u>
30-Antisense	<u>ATTTAGGTGACACTATAGAAAATATGTTATTTACCTTTGG</u>
31-Sense	<u>TAATCGACTCACTATAGGGCTTCTTGTATCATGGATGTG</u>
31-Antisense	<u>ATTTAGGTGACACTATAGAAAAATTACCTGTTTCTGAACC</u>
32-Sense	<u>TAATCGACTCACTATAGGGTTTTCTTTTTAAATTATATT</u>
32-Antisense	<u>ATTTAGGTGACACTATAGAATTTATTACCTCCAAGAG</u>
33-Sense	<u>TAATCGACTCACTATAGGGATTAACCATTTTCTCTCAGT</u>
33-Antisense	<u>ATTTAGGTGACACTATAGAAAAGCTCTCATAATGTCCA</u>
34-Sense	<u>TAATCGACTCACTATAGGGAATTATTTCTAGATAATCCG</u>
34-Antisense	<u>ATTTAGGTGACACTATAGAAGCATGACTGTAGTTTACCTA</u>
35-Sense	<u>TAATCGACTCACTATAGGGGAATTTCTATATGTAGAGGC</u>
35-Antisense	<u>ATTTAGGTGACACTATAGAAGGTATCAATAAATACTCACC</u>
36-Sense	<u>TAATCGACTCACTATAGGGTTTTCAGTGTCAAAGTTCTG</u>
36-Antisense	<u>ATTTAGGTGACACTATAGAATTCTTGATGTTCTAAAAGGC</u>
37-Sense	<u>TAATCGACTCACTATAGGGTCTAGTTTTTAGAAGTACCC</u>
37-Antisense	<u>ATTTAGGTGACACTATAGAAGGCTTTAATAATTGAAGAA</u>
38-Sense	<u>TAATCGACTCACTATAGGGGGGTGGATTTGTTTGT</u>
38-Antisense	<u>ATTTAGGTGACACTATAGAAATAGAATACCTGAATCCAAG</u>
39-Sense	<u>TAATCGACTCACTATAGGGGTTTGTCAGAGTCAGAGCA</u>
39-Antisense	<u>ATTTAGGTGACACTATAGAACCTCTTTTGTCTTCTCATGT</u>
40-Sense	<u>TAATCGACTCACTATAGGGCTCATTTTTCTTTAGACCTT</u>
40-Antisense	<u>ATTTAGGTGACACTATAGAACAAAAATTCTAAATTCCATT</u>
41-Sense	<u>TAATCGACTCACTATAGGGTTGTGACAAACAGAAGTC</u>
41-Antisense	<u>ATTTAGGTGACACTATAGAAGTCATAAATAATAGAGCCTC</u>
42-Sense	<u>TAATCGACTCACTATAGGGTGTTTTCAGGATCTTCTCTT</u>
42-Antisense	<u>ATTTAGGTGACACTATAGAATGTAACATCTTCCCTCCA</u>



43-Sense	<u>TAATCGACTCACTATAGGGCTACGAACATATGAACACGA</u>
43-Antisense	<u>ATTTAGGTGACACTATAGAAAAATGTACCTCAATGATTCC</u>
44-Sense	<u>TAATCGACTCACTATAGGGGCCTTGCAGAATTTGGGACT</u>
44-Antisense	<u>ATTTAGGTGACACTATAGAACTGACGGAAGTGCAATGGTC</u>
45-Sense	<u>TAATCGACTCACTATAGGGAGCAAAGAAGTAGAAGGAAC</u>
45-Antisense	<u>ATTTAGGTGACACTATAGAATAATACCTGGCATATTTGAG</u>
46-Sense	<u>TAATCGACTCACTATAGGGCATAGAGTAAAAGAAGTGGA</u>
46-Antisense	<u>ATTTAGGTGACACTATAGAATACATACCTTGAGAAAAGC</u>
47-Sense	<u>TAATCGACTCACTATAGGGGCATTTCAGATCAGTCACAC</u>
47-Antisense	<u>ATTTAGGTGACACTATAGAAATTTACCTGAGTGTTCTTGA</u>
48-Sense	<u>TAATCGACTCACTATAGGGAAGGGCAATATTTCAAATTA</u>
48-Antisense	<u>ATTTAGGTGACACTATAGAACAACCGCTGCACAG</u>
49-Sense	<u>TAATCGACTCACTATAGGGTTTTAAGATTTTGCCTTTCT</u>
49-Antisense	<u>ATTTAGGTGACACTATAGAACTTAAGGGTTGCTCCAA</u>
50-Sense	<u>TAATCGACTCACTATAGGGTTCTTGAAGGCAGTAGAAGT</u>
50-Antisense	<u>ATTTAGGTGACACTATAGAAATGTCTGAATTTTATGTTCCC</u>
51-Sense	<u>TAATCGACTCACTATAGGGTGCAAGATACACAGTAAAGG</u>
51-Antisense	<u>ATTTAGGTGACACTATAGAATCATCATGCCATTGACTTCA</u>
52-Sense	<u>TAATCGACTCACTATAGGGTGAAGATTCCAACATATAAA</u>
52-Antisense	<u>ATTTAGGTGACACTATAGAAATGTGGTTTGATTTTCAG</u>
53-Sense	<u>TAATCGACTCACTATAGGGAATCTCTAGAATTTCAATGG</u>
53-Antisense	<u>ATTTAGGTGACACTATAGAAAAGGTACGTATGTTTAATCC</u>
54-Sense	<u>TAATCGACTCACTATAGGGTTTTATTAATAGGATCGAA</u>
54-Antisense	<u>ATTTAGGTGACACTATAGAAAAAACATACTTCTCTGAGT</u>
55-Sense	<u>TAATCGACTCACTATAGGGCTTCATTTTTAAATACAGAA</u>
55-Antisense	<u>ATTTAGGTGACACTATAGAAAAATCAAGAGTTAATTGC</u>
56-Sense	<u>TAATCGACTCACTATAGGGTTTATACTTTTATTAGGTGG</u>
56-Antisense	<u>ATTTAGGTGACACTATAGAAGCTAAGCCAGAGAAGG</u>
57-Sense	<u>TAATCGACTCACTATAGGGTTGTTTATTCATGCTTAAT</u>

57-Antisense	<u>ATTAGGTGACACTATAGAATGAACTAACAGAAGTACAAA</u>
58-Sense	<u>TAATCGACTCACTATAGGGCTCAGCGAAGTGGTGTTT</u>
58-Antisense	<u>ATTAGGTGACACTATAGAAATCATTTTCTTTGGCACTG</u>
59-Sense	<u>TAATCGACTCACTATAGGGATATTCTCTATTTAAAGGAG</u>
59-Antisense	<u>ATTAGGTGACACTATAGAATATGTACAAGAAGATTACC</u>
60-Sense	<u>TAATCGACTCACTATAGGGCAGATTGTTTGTTTCTT</u>
60-Antisense	<u>ATTAGGTGACACTATAGAACATAGATTTTATTACTTACC</u>
61-Sense	<u>TAATCGACTCACTATAGGGCTTTACTTTAGGTGTTGCTT</u>
61-Antisense	<u>ATTAGGTGACACTATAGAACTTACCTTCTGAAGACA</u>
62-Sense	<u>TAATCGACTCACTATAGGGCCGTATTTATAATGTGTT</u>
62-Antisense	<u>ATTAGGTGACACTATAGAATCTTCCTTATAAAATACTTT</u>
63-Sense	<u>TAATCGACTCACTATAGGGAGGTCCTTCTATATGATCCA</u>
63-Antisense	<u>ATTAGGTGACACTATAGAAACAACAGGACCTTCTTAAAA</u>

The underlined regions indicate the phage promoter sequences.

*Breast Cancer Research and Treatment*

***ATM* heterozygosity and breast cancer: screening of 37 breast cancer patients for *ATM* mutations using a non-isotopic RNase cleavage-based assay**

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This paper is dedicated to the memory of Eva Levine, whose extraordinary sensitivity and devotion to her patients was an inspiration to her colleagues and will forever be missed.

## Summary

Based upon the results of several epidemiologic studies, it has been suggested that women who are carriers for a mutation in the ataxia telangiectasia-mutated (*ATM*) gene are susceptible for the development of breast cancer. Therefore, 37 consecutive breast cancer patients were screened for the presence of a germline *ATM* mutation using a non-isotopic RNase cleavage based assay (NIRCA). This paper reports the first use of NIRCA for detection of *ATM* mutations in breast cancer patients. Using this assay, no *ATM* mutations were found in our patient population. This result is similar to the findings of other studies that have employed approaches complementary to NIRCA. Therefore, although the size of the population examined was modest, resulting in a relatively low power for this study, this finding is consistent with the conclusion that *ATM* carriers may not be at greater risk for breast cancer compared with women not possessing a mutation in this gene.

**Key Words:** breast cancer, ataxia telangiectasia, *ATM*, mutation screening

## Introduction

Ataxia-telangiectasia (AT) is an autosomal recessive disorder characterized by progressive cerebellar degeneration with ataxia, conjunctival telangiectasias, immunodeficiency, chromosomal instability, radiation sensitivity and cancer predisposition [1]. The cancer risk exhibited by AT patients is believed to be approximately 60-180 times higher than that of the general population [2], consisting primarily of lymphomas and leukemias. *ATM*, the gene whose alteration results in AT, has been cloned and mapped to chromosome 11q22-23 [3, 4]. *ATM*, which is expressed in a wide variety of tissues, is approximately 150 kb in length possessing 66 exons with a 12 kb transcript. The open reading frame of 9168 nucleotides encodes a nuclear phosphoprotein of 3056 amino acids. Most AT patients are compound heterozygotes with the location of mutations spread throughout the genome [5]. Concannon et al. [6] found that 72% of 78 unique mutations demonstrated in AT patients would be predicted to produce either a truncated protein or no product.

A hallmark of this disease is great sensitivity to radiation as AT patients who were exposed to conventional radiation doses for cancer treatment developed devastating tissue necrosis [7]. The basis for this radiosensitivity has long been the subject of investigation that advanced significantly with cloning of the *ATM* gene. It has been demonstrated that the ATM protein belongs to an expanding family of large eukaryotic proteins involved in intracellular signaling, cell cycle control, DNA repair and recombination in response to DNA damage. This family of proteins has been grouped together with *ATM* primarily based

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upon the strong homology of their carboxyl termini to the 100 kDa catalytic subunit of the mammalian signal transduction mediator phosphatidylinositol-3 (PI-3) kinase. PI-3 kinases appear to participate in many cellular processes, including insulin-dependent glucose transport, growth factor responses and cellular differentiation [8-10].

The current explanation for the function of the *ATM* gene product is that it has been linked to the p53 signal transduction pathway responsible for cell cycle arrest [11] following DNA damage. Several studies have revealed that cells derived from AT patients exhibit defective or delayed increases in p53 following irradiation, suggesting that normal *ATM* function is required for optimal transduction of the signal from the initial radiation-induced DNA damage to the modulators of the increase in p53 levels [12]. AT cells are also defective in their ability to induce downstream genes, including *GADD45*, *p21* and *MDM2* [12]. It has been shown that the AT defect is translated into abrogation of the G1, S and G2-M checkpoints of the cell cycle [13].

The worldwide incidence of AT is estimated at 1:40,000-1:100,000 live births [14, 15]. In contrast to the relative rarity of this disease resulting from *ATM* homozygosity, heterozygosity is far more common and it has been estimated that approximately 1 percent of the population possesses a mutation in one copy of this gene [16]. These heterozygotes do not manifest any outward radiation sensitivity, but, cells derived from individuals who are carriers for a mutation in this gene exhibit a slightly increased radiation sensitivity [17, 18].

Based upon the results of epidemiologic studies, it has been suggested that *ATM* carriers have a 2-6 fold greater risk for cancer development and are

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particularly predisposed to breast cancer compared with non-carriers [19-21]. It has been estimated that 9-18 percent of all women with breast cancer may be *ATM* heterozygotes [22]. To address this issue, Easton [23] performed a pooled analysis of four studies [19, 20, 22, 24] from which it can be suggested that *AT* heterozygotes are more prone to breast cancer and could account for up to 13% of breast cancer cases, with 3.8% being the best estimate. However, recent studies have cast a doubt upon this suggestion as the level of mutations detected among breast cancer patients has been consistent with only the 1% frequency estimated for the general population [25-27]. In addition, *ATM* mutations have not been detected in breast cancer patients who developed unusually severe responses to radiotherapy indicating that *ATM* heterozygosity does not result in acute complications associated with this treatment [28-30].

All of the studies performed to date have been accomplished using either the protein truncation test (PTT), restriction-endonuclease fingerprinting (REF) or single strand conformation polymorphism (SSCP). However, it is important to note that none of these techniques is capable of detecting all mutations [7] and it is always essential to use a series of screening methods to locate most mutations in a population. It is therefore important to employ a complementary assay with a different basis to identify mutations which had possibly gone undetected using other techniques. NIRCA represents such an alternative approach. Hence, the purpose of this study was to determine the prevalence of germline *ATM* mutations in breast cancer patients using NIRCA.

## Methods

### *Patients*

Three ml peripheral venous blood samples were obtained from a hospital-based series of 37 unselected patients diagnosed with primary breast cancer treated with radiation therapy with or without chemotherapy between 1992 and 1997. The nature of the project was discussed with each patient and signed informed consent was obtained prior to obtaining each blood sample. Patient characteristics were obtained by chart review and are presented in Table 1.

### *RNA extraction and cDNA synthesis*

Lymphocytes were isolated by Ficol-plaque separation. 1.5 ml Ficol was added to 3 ml of blood and centrifuged at 12000 rpm for 20 min at room temperature. The lymphocytes layer was collected, washed with 10 ml PBS and centrifuged for 10 min, room temperature, at 12,000 rpm. To isolate RNA from the lymphocytes, 2 ml RNAzol™ (Cinna/TEL-TEST) was added to the cells, followed by addition of 100 µl of chloroform and centrifuged at 4°C for 15 min at 12,000 rpm. The supernatant was transferred to a 1.5 ml tube and an equal volume of isopropanol added to the supernatant. The tube was held at -20°C overnight and centrifuged 15 min at 4°C. The supernatant was removed and the pellet washed with 500 µl of 70% ethanol. After centrifugation at 4°C, 12,000 rpm, for 8 min, the supernatant was removed and the pellet washed with 200 µl of 70% ethanol



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and centrifuged once more at 4°C, 12,000 rpm speed, for 8 min. The supernatant was removed, the pellet air-dried and 30 µl RNase free H<sub>2</sub>O added.

cDNA was synthesized using the isolated RNA as a template by combining 20 µl RNA solution with 4 µl random primers. The mixture was heated to 70°C for 10 min and quickly chilled on ice. After a brief centrifugation, 4 µl of 5X first strand buffer, 2 µl 0.1 M DTT and 1 µl 10mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP at pH 7.0) were added and the mixture incubated 2 min at 42°C. 2 µl (200 units) SUPERScript™ was added, the mixture incubated 50 min at 42°C and then 70°C for 15 min. To remove RNA complementary to the cDNA, the reaction was treated with 2 units E. coli RNase H and incubated at 37°C for 20 min.

#### *PCR conditions and primers*

Segments of the *ATM* gene were amplified from aliquots of the cDNA template using sets of primer pairs. The primers were designed with the aid of Oligo-5 software (NBI, Plymouth, MN) and are listed in Table 2 (first stage primers) and Table 3 (second stage primers). The *ATM* open reading frame was amplified in a series of 12 overlapping fragments that were approximately 1 kb in size. The product of each PCR reaction was then used as a template for a second reaction to further amplify this region of the gene. In addition, the primers were designed so that sequences for the T7 and SP6 promoters were incorporated into the 5' end of each sense and antisense primer, respectively. 2 µl of cDNA was used in a 50 µl PCR reaction using 250 nM first stage primers, 125 µM dNTPs, 1 U Taq

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polymerase in a buffer containing 10 mM TrisCl at pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 0.01% gelatin. The PCR reactions were performed in a Perkin Elmer 9700 ThermalCycler with the following settings: 94°C for 4 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and one cycle for 7 min at 72°C. One µl of first stage PCR product was used in the nested PCR reactions using the same conditions, with second stage primers.

### *Non-Isotopic RNase Cleavage Assay (NIRCA)*

The MisMatch Detect II Non-Isotopic RNase Cleavage Assay Kit (Ambion, Austin, TX) was used to screen the samples for *ATM* mutations [31, 32]. Each second stage PCR product was transcribed in two reaction mixes using 4 µl of PCR product, 250 nM rNTPs, 1 U of either T7 or SP6 RNA polymerase in a buffer containing 40 mM TrisCl, pH 7.5, 7 mM MgCl<sub>2</sub>, 2 mM spermidine, 25 mM NaCl and 10 mM dithiothreitol and brought to a final volume of 12 µl with RNase free H<sub>2</sub>O. Following incubation for 90 min at 37°C, 6 µl of buffer containing 80% formamide, 25 mM NaCl, 2mM EDTA, pH 8.0, was added to each transcription reaction. The T7 and SP6 reactions were mixed, incubated at 95°C for 5 min and allowed to cool at room temperature for 5 min. 4.5 µl of the duplex reactions was mixed with 13.5 µl of RNase digestion buffer (10 mM NaCl, 10mM TrisCl, pH 7.5, 1mM disodium EDTA, pH 8.0) with RNase 2 and RNase 1 (from *E. coli*) at a concentration of 1:100 (0.5 ug/ml) and 1:200, respectively. The reaction mixtures were incubated at 37°C for 25 min and resolved on a 2% agarose gel run at 100V for approximately 45 min.

## Results

RNA was isolated from peripheral blood lymphocytes of 37 breast cancer patients in order to detect possible *ATM* mutations. The characteristics of the subjects are presented in Table 1. cDNA copies of the extracted mRNA were produced for each sample. This approach was employed, rather than the use of genomic DNA, to avoid analysis of intronic DNA regions. The *ATM* open reading frame was amplified in 11 overlapping segments using nested PCR. The products of the second stage PCR reactions, all roughly 1 kb in size, were subjected to agarose gel electrophoresis and the results obtained for 3 patients using 2 sets of primers and a *p53* control displayed in Fig.1.

The PCR products that contained promoters for either the T7 or SP6 RNA polymerase were then transcribed in an in vitro system. The convention followed with this assay was linkage of the T7 promoter to the sense strand of the fragment while the SP6 promoter was attached to the antisense strand. The RNA product produced from either the sense or antisense strand was hybridized with the complementary RNA strand produced using known normal tissue not possessing an *ATM* mutation. In addition, for each experiment, the RNA produced from a known mutant and wild type was hybridized with RNA transcribed from a wild type DNA template to serve as positive and negative controls for this assay, respectively.

These duplexes were subjected to agarose gel electrophoresis either prior to (Figure 2) or following digestion (Figure 3) with RNase. The basis of this screening technique is the sensitivity to cleavage with RNase of an RNA duplex

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containing a mismatched region [31, 32]. This heteroduplex forms when RNA produced from a PCR product possessing a mutation at a particular site is hybridized with RNA produced from a wild type DNA template. As can be seen in figure 3, there was the appearance of two bands following digestion of the RNA duplex derived from DNA fragments containing a known mutation in the *p53* gene confirming the presence of a mutation. In contrast, none of the assays performed to detect *ATM* mutations exhibited multiple bands indicating that there were no *ATM* mutations present in the breast cancer patients tested.

## Discussion

A series of 37 breast cancer patients was screened for germline mutations in the *ATM* gene. This work was accomplished using NIRCA, an assay that is complementary to the approaches which have been previously used to estimate *ATM* mutation frequency in breast cancer patients. No *ATM* mutations were detected in this study, which is consistent with the results obtained using other techniques [25-27]. Although the number of patients tested in this study was modest, our finding of no *ATM* mutations in a group of 37 patients provides a 95% upper confidence interval limit for *ATM* carriers among breast cancer patients of 7.8%.

NIRCA was used in this study for a variety of reasons: 1) It has been shown that NIRCA is capable of detecting a variety of mutations, including point mutations as well as deletions and insertions, with a high level of sensitivity and accuracy [31-41]; 2) Relatively large target regions of at least 1000 bp can be screened in a single step. This is in contrast to other assays that are limited to much smaller target regions. This is critical for screening *ATM*, as even the expressed portion of the gene is large, and the identified mutations are scattered throughout the gene; 3) Using NIRCA, it is not necessary to possess any information as to potential sites for mutations. The entire coding sequence of the gene can be analyzed and therefore mutations at any point along the gene can be detected; 4) The position of the bands on a gel provide a preliminary identification of the location for a mutation which facilitates DNA sequencing and analysis of sequencing data.

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**Table 1.** Breast cancer patient characteristics

Patient characteristics	Number of patients (%)
<b>Age Group (years)</b>	
Under 40	2 (5.5)
40-59	14 (37.8)
60-80	20 (54)
Over 80	1 (2.7)
Median Age = 62	
<b>Ethnic group</b>	
Caucasian <sup>a</sup>	20 (54)
Hispanic	13 (35.2)
Black	3 (8.1)
Asian	1 (2.7)
<b>Menopausal status</b>	
Pre- and peri-menopausal	9 (24.3)
Postmenopausal	28 (75.7)
<b>Family history of cancer</b>	
Breast <sup>b</sup>	13 (35.2)
Other site	9 (24.3)
None	15 (40.5)

## Drumea

### Stage of disease

Stage 0	3 (8.1)
Stage I	27 (80)
Stage II	6 (16.2)
Stage III	1 (2.7)

### Histology

Intraductal	3 (8.1)
Infiltrating duct	26 (70.1)
Infiltrating lobular	2 (5.5)
Tubular	1 (2.7)
Tubulo-lobular	3 (8.1)
Adenocarcinoma	2 (5.5)

<sup>a</sup> 11 patients of Ashkenazi Jewish background

<sup>b</sup> 7 patients with first-degree relatives

Table 2. Sequences of first stage *ATM* primers

Fragment Number	Bases Amplified	Primer Sequence
1-Sense	5-906	GAGGAGTCGGGATCTGC
1-Antisense		GACAGCCAAAGTCTTGAG
2-Sense	695-1676	CTGTGTACTTCAGGCTCT
2-Antisense		GCTTGTATTTGCTCAGAA
3-Sense	1544-2509	CACCATATGTGTTACGAT
3-Antisense		TCATATTTCTCAAGGAAC
4-Sense	2329-3298	ACTCTTGTCCGGTGTTT
4-Antisense		AGGGCCATTCTTACAGA
5-Sense	2978-3977	TAATTGATTCTAGCACGC
5-Antisense		CTTCTAATCACCAGATGT
6-Sense	3877-4850	AACTTATCTTCTTTTCC
6-Antisense		AATCGTGATATAGAGGT
7-Sense	4710-5682	TTACTGTAAGGATGCTC
7-Antisense		AGTCAGTTTTCACTTCA
8-Sense	5559-6534	TGATATAAATCTGTGGA
8-Antisense		TTCTACTTCTTTGCTG
9-Sense	6407-7353	TCTGCCATATTCTTTCC
9-Antisense		AACCGGGCTAATGAG
10-Sense	7193-8172	CAGAATGTCTGAGGGT
10-Antisense		TAGTAGGGACAACAACA

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11-Sense	8047-8952	GTTGAGGCACTTTGTGA
11-Antisense		AGACACCTTCAACACCC
12-Sense	8409-9353	GAGAAACACGGAAACTA
12-Antisense		TACTGAAGATCACACCC



Table 3. Sequences of second stage *ATM* primers

Fragment Number	Bases Amplified	Primer Sequence <sup>a</sup>
1-T7	37-790	<u>TAATCGACTCACTATAGGG*</u> CGGTTGATACTACTTTG
1-SP6		<u>ATTTAGGTGACACTATAGAA</u> TTTAATCCGTCAGTCT
2-T7	755-1632	<u>TAATCGACTCACTATAGGA</u> ATAATTCATGCTGTTAC
2-SP6		<u>ATTTAGGTGACACTATAGAA</u> TTTTATTCCAGAGTTT
3-T7	1570-2425	<u>TAATCGACTCACTATAGGA</u> AGTTGCATTGTGTCAAG
3-SP6		<u>ATTTAGGTGACACTATAGGA</u> GTTGGCTTTCTGGAA
4-T7	2368-3247	<u>TAATCGACTCACTATAGGG</u> CTGCTACTGTTACA
4-SP6		<u>ATTTAGGTGACACTATAGAA</u> TGCTCCAATTACTGT
5-T7	3055-3944	<u>TAATCGACTCACTATAGGA</u> GAAGAGTACCCCTTGC
5-SP6		<u>ATTTAGGTGACACTATAGGA</u> GATGTGGAATCAAAACCTTAT

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6-T7	3911-4796	<u>TAATCGACTCACTATAGGA</u> CACAAATATTGAGGAT
6-SP6		<u>ATTTAGGTGACACTATAGAA</u> GTCCAATACCTGTT
7-T7	4782-5644	<u>TAATCGACTCACTATAGGG</u> TGGAGGTTTCAGA
7-SP6		<u>ATTTAGGTGACACTATAGAA</u> GAATTTACATTTTGT
8-T7	5607-6500	<u>TAATCGACTCACTATAGGA</u> AAGACACTGACTTGTG
8-SP6		<u>ATTTAGGTGACACTATAGAA</u> CTGCATATTCCTCC
9-T7	6451-7285	<u>TAATCGACTCACTATAGGA</u> ATAAAGACTGGTGTCC
9-SP6		<u>ATTTAGGTGACACTATAGAA</u> TTTCCAGCAACTTC
10-T7	7213-8113	<u>TAATCGACTCACTATAGGG</u> CAACTGGTTAGC
10-SP6		<u>ATTTAGGTGACACTATAGAA</u> TATTTATGCCTTTTCT
11-T7	8082-8669	<u>TAATCGACTCACTATAGGA</u> AACTTAGATGCCACTC
11-SP6		<u>ATTTAGGTGACACTATAGGA</u> AAACTGGTTGAA

12-T7

8598-9328

TAATCGACTCACTATAGGA

AAATGATGGAGGTGC

12-SP6

ATTTAGGTGACACTATAGAA

TCCTGGGAAAAGTCG

<sup>a</sup> The underlined regions indicate the phage promoter sequences. Generally, the first two bases at the 3' end of the promoter overlapped in each case with the first two bases of the *ATM* fragment.

## Figure Legends

*Figure 1. PCR Products.* Second stage PCR products for DNA isolated from two breast cancer patients (lanes 1 and 2) and one person not diagnosed with breast cancer (lane 3) and amplified with primers for Fragments 4 and 6, labeled as A and B, respectively. DNA known not to possess a mutation in the *p53* gene and a DNA sample with a known mutation are shown in lanes 4 and 5, respectively. These samples were amplified with primers for a DNA fragment encompassing *p53* exons 5 and 6 and labeled C.

*Figure 2. Transcription Products – No Digestion.* RNA products resulting from hybridization of RNA produced from the sense strand of tumor biopsy DNA and RNA synthesized from the antisense strand of DNA isolated from normal human DNA. The lanes are labeled as described in Figure.1.

*Figure 3. RNase-Treated Transcription Products.* Products resulting from RNase digestion of the duplex RNAs shown in Figure 2.

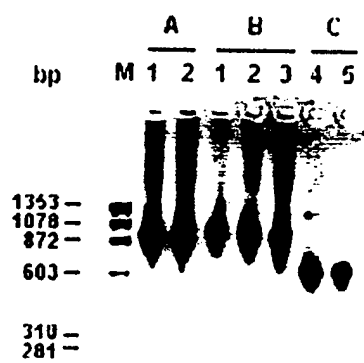


Figure 1

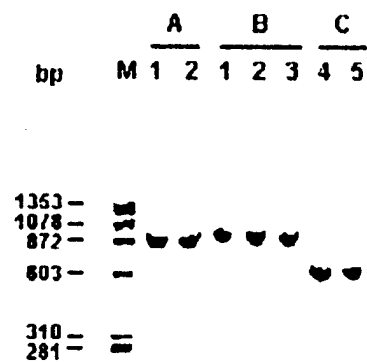


Figure 2

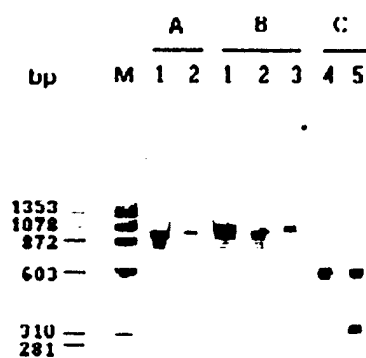


Figure 3